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Onset of ATP synthesis in spinach chloroplasts after single-turnover flashes in relation to adenylate kinase activity

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Flash-induced ATP synthesis was investigated in chloroplasts isolated from spinach varieties with a pronounced difference in their adenylate kinase activity. In chloroplasts with high adenylate kinase activity the first 4 flashes induced ATP hydrolysis which persisted for an extended dark period at which the membrane remained energized. In chloroplasts showing low adenylate kinase activity, ATP hydrolysis could only be induced by a successive flash train of at least 60 flashes or by a 30–60 s period of continuous pre-illumination. The activity of the adenylate kinase must be considered when the kinetics of initial ATP synthesis and of P515 in light flashes are studied.

ATP synthesis *ATP hydrolysis* *Single-turnover flash* *Thylakoid membrane* *ATPase* *P515* *Electrochromic bandshift* *Adenylate kinase* *Enzyme activity*

1. INTRODUCTION

Direct monitoring of flash-induced ATP synthesis in chloroplasts has been reported by Schreiber and Del Valle-Tascon [1]. Using the firefly luciferase method both ATP synthesis and hydrolysis could be measured simultaneously in the same chloroplast suspension. These experiments showed that in fully dark-adapted chloroplasts the first 6 flashes induced ATP consumption and only after a certain period of ATP hydrolysis was net ATP synthesis observed. The onset of initial ATP consumption, following 8 h of strict dark adaptation, could be shown to be triggered by as little as 2 single-turnover saturating actinic flashes given 1 s apart [1]. Accordingly, Graan et al. [2] reported flash-induced ATP synthesis in chloroplasts using a sensitive ^{32}P method. In agreement with Schreiber and Del Valle-Tascon, these authors found flash-induced ATP yields provided 5 activating flashes were given. Unfortunately, their method did not allow monitoring of ATP synthesis parallel to ATP hydrolysis. However, ATP hydrolysis has been shown to occur in intact and freshly broken chloroplasts and has been reported to pertain for extended periods of darkness after light activation of the reversible ATPase [3–6]. The activated membrane-bound ATPase is known to invoke a considerable electrochemical potential gradient of protons ($\Delta\mu H$) [7–12]. This gradient that is present in the dark as a result of ATP hydrolysis can be considered as a 'threshold' energy for ATP synthesis. Consequently, even small increments of the proton-motive force could result in the formation of ATP under these conditions. This may explain the rate of ATP synthesis found in [1] which increased with the number of flashes to a quasi-stationary value of 50 nmol ATP $\cdot$ mg^{-1} Chl $\cdot$ min^{-1} and equivalent to about 1 ATP/CF_1 per flash. The same optimal yield of flash-induced ATP synthesis was found in [2]. The effect of the first activating flashes on the reversible ATPase will be dependent on the value of ΔG_{ATP} which is affected by adenylate kinase ac-

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tivity. When there is a high adenylate kinase activity, as for instance in the experiments reported in [1], addition of ADP will result in the conversion of a significant amount of ADP into ATP in the dark. This dark conversion will clearly affect ΔG_{ATP} and will favour the conditions for ATP hydrolysis by the reversible ATPase.

To study the effect of the first activating flashes on the reversible ATPase in dark-adapted chloroplasts in relation to the adenylate kinase activity, we compared these effects for two types of chloroplasts which showed a pronounced difference in endogeneous adenylate kinase activity. It is shown that in spinach chloroplasts of a summer variety with a high adenylate kinase activity the first 4 flashes result in ATP hydrolysis. In chloroplasts of a winter variety with a low activity of the enzyme at least 60 flashes are needed. In both types of chloroplasts, flash-induced ATP synthesis was about 1 molecule ATP/CF_1 per flash provided the ATPase was pre-activated. The energy state of the thylakoid membrane after activation of the ATP hydrolase was reflected in the kinetics of the flash-induced P515 electrochromic bandshift as reported earlier [14]. These experiments demonstrate that the length of the pre-illumination period (i.e. the number of activating flashes) needed to activate the reversible ATPase is dependent on the value of ΔG_{ATP}. This potential is affected by the activity of the adenylate kinase enzyme. A high activity of the enzyme will result in conditions that are favourable for ATP hydrolysis by the membrane-bound ATPase. Since this hydrolysing activity of the ATPase will result in energization of the membrane, and in this way contribute to the energetic state which determines light-driven ATP production, adenylate kinase activity must be taken into account when studies of flash-induced ATP synthesis and of P515 kinetics are performed.

2. MATERIALS AND METHODS

Freshly grown spinach (*Spinacia oleracea*) was used for all experiments. The plants were grown in a greenhouse under high-pressure mercury lamps (Philips MGR 102-400) at an intensity of approx. 100 $W \cdot m^{-2}$ with a light period of 8 h per day. Provisions were made to keep the temperature at the leaf and soil surface at 18–20°C. The relative humidity of the atmosphere was minimally 70%. Two different spinach cultivars were used. One cultivar (cv. Nobel), known as a summer variety, has been found to require a light intensity of at least 200 $W \cdot m^{-2}$ for optimal growth. The other (cv. Bergola), a winter variety, grows optimally at a light intensity of about 80 $W \cdot m^{-2}$. Both types of plants were grown under identical environmental conditions. The procedures used for the isolation of chloroplasts were also the same. Intact chloroplasts routinely were isolated according to a modified method of [15] as described in [16]. This procedure yielded preparations with 90–95% intact chloroplasts as determined by ferricyanide reduction [17]. Following isolation, chloroplasts were stored in the dark at 0°C at a chlorophyll concentration of approx. 1.0 mg/ml. Shortly before each experiment 60 μl chloroplasts were osmotically ruptured by mixing with 1 ml hypotonic medium consisting of 5 mM Hepes-KOH (pH 7.5), 5 mM Mg acetate and 4 mM DTE. After 60 s incubation the suspension was made half-isotonic by the addition of 1 ml of the following buffer: 330 mM sorbitol, 20 mM Hepes-KOH (pH 7.5), 2 mM NaH_2PO_4, 2 mM Mg acetate and 2 mM DTE. Final chlorophyll concentration was 30 μg/ml.

The chloroplast suspension was continuously stirred in a 3 ml cuvette thermostatted at 10°C. ATP concentration changes were measured with the luciferin-luciferase luminescence assay in an appropriate monitoring device [18], equipped with a photomultiplier. For each experiment 200 μl of a solution of 'ATP monitoring reagent' (LKB Wallac, the vial content being dissolved in 10 ml double-distilled water) was added to the cuvette. ADP (Boehringer) was added at 20 μM. ATP calibration was done with known amounts of a freshly prepared 10 μM ATP solution (LKB Wallac). Saturating single-turnover actinic flashes with a half-width of 8 μs were transmitted to the sample via light guides. Pre-illumination with red light came from a 250 W tungsten lamp and was transmitted to the sample via light guides. The photomultiplier was shielded from actinic light by an appropriate filter combination. Absorbance changes at 518 nm, induced by single-turnover flashes, were measured in a modified Aminco-Chance absorption difference spectrophotometer as in [14].

3. RESULTS AND DISCUSSION

The left-hand part of fig.1A,B shows the luciferin luminescence response upon addition of 20 μM ADP to samples of dark-adapted chloroplasts isolated from the summer (A) and winter (B) spinach variety, respectively. A biphasic rise of luminescence upon addition of ADP is obvious in both samples. The rapid phase (about equal in both samples) is due to contaminating ATP (0.1%) in the ADP solution. The slow phase is due to the action of adenylate kinase. There appears to be a pronounced difference with respect to the enzyme activity between the two chloroplast preparations. This activity can be calculated using the second-order rate constant of the enzymic reaction and the amount of ADP converted. In summer chloroplasts, at the concentration of ADP used, the data of fig.1A yield a rate constant of about 144 $M^{-1} \cdot s^{-1}$. From fig.1A it can be seen that in the summer spinach variety about 50% of the amount of added ADP has been converted into ATP in the dark due to this activity. Approximately the same kinase activity and rate of ADP conversion can be calculated from the data in fig.1 in [1]. Under exactly the same experimental conditions, the activity of the adenylate kinase enzyme in winter chloroplasts can be calculated to be about

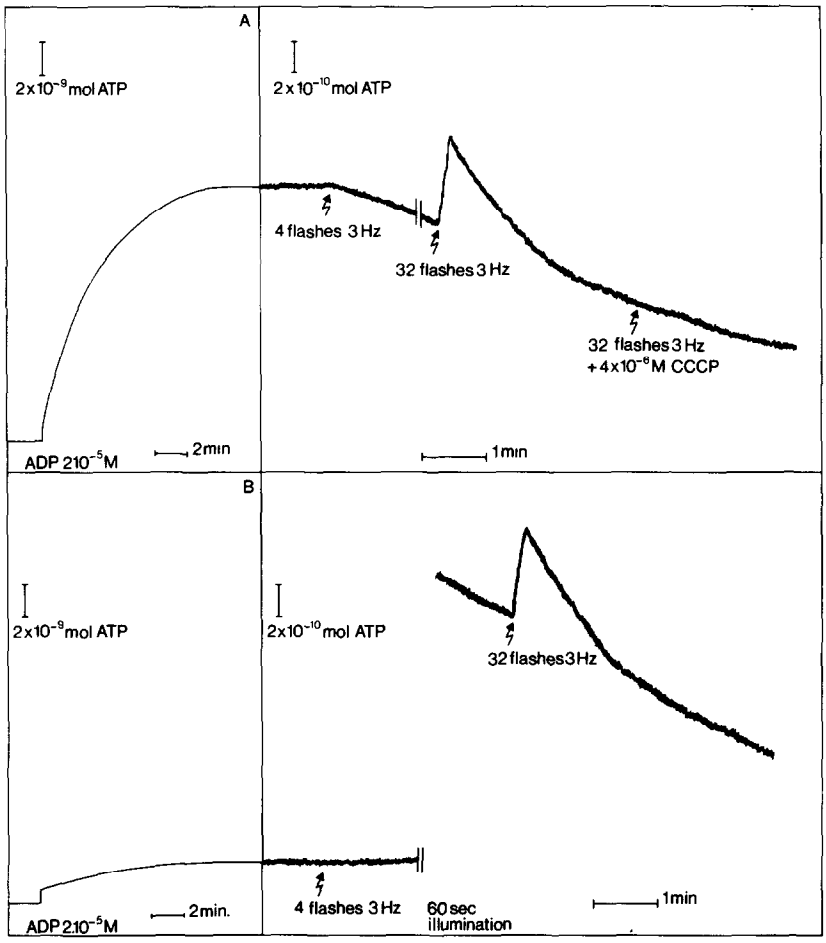

Fig.1. Monitoring of ATP with the firefly luciferase method in chloroplasts isolated from a summer (A) and a winter (B) spinach variety. (Left) Response due to adenylate kinase activity upon addition of 2×10^{-5} M ADP. (Right) Luminescence response following illumination of chloroplasts with successive flash trains of 4 and 32 flashes, determined on a 10-times more sensitive scale.

$8.4\ M^{-1} \cdot s^{-1}$ (fig.1B). In these chloroplasts, the amount of ADP converted into ATP in the dark was found to be much less (maximally 10%, fig.1B).

This conversion of added ADP into ATP prior to illumination will affect ΔG_{ATP}, favouring ATP-hydrolysing conditions. As a result, conditions in favour of ATP hydrolysis will be more pronounced in the sample of chloroplasts isolated from summer type spinach. After the kinase-dependent dark reaction was completed, 4 saturating flashes were given at 3 Hz repetition rate every 4 min and the luminescence signal was monitored on a 10-times more sensitive scale. The right-hand side of fig.1A shows that in summer chloroplasts the first series of four flashes induce ATP hydrolysis in the subsequent dark period. In accordance with results of others [1,14] it was found that this hydrolysis of ATP continued for an extended dark period. It has been reported [14] that ATP hydrolysis in the dark results in energization of the thylakoid membrane. This energization has been shown to be reflected by altered kinetics of the flash-induced P515 electrochromic bandshift [14]. The slow phase (i.e. reaction 2), contributing to the overall signal in dark-adapted chloroplasts is virtually absent under energized conditions of the membrane. It has been suggested by others [19–22] that reaction 2 results from redox reactions in the Qbc region of the electron transport chain. An association with cytochrome *b*-563 reoxidation has been suggested [23]. However, it has been shown [24] that the seeming correspondence between the potential associated with this secondary electron transport and a turnover of cytochrome *b*-563 only holds for a single flash. A second turnover of cytochrome *b*-563 induced by a second flash, given 100 ms after the first one, does not, or only to a much smaller degree, cause a slow rise in the flash-induced P515 response. Moreover, the suggested association of the slow phase in the rise of the P515 response with secondary electrogenetic electron transport does not account for the fact that the field generated by this electron transfer decays with a half-time which is considerably higher than the decay of the potential generated by electron transfer through the reaction centers [25]. On the basis of the kinetics of reaction 2 we suggest (in conformation with others [12,25]) that reaction 2 is caused by lateral and transverse delocalization of inner-membrane electric fields associated with the liberation and subsequent stabilization of protons in inner-membrane domains near the Fe-S cytochrome *b-f* protein complex. From fig.2A it can be seen that in summer chloroplasts after 4 activating flashes, the kinetics of the flash-induced P515 bandshift indeed is devoid of the slow phase normally present in dark-adapted membranes. A second flash train (32 flashes), given 4 min after the first 4 activating flashes, caused a net synthesis of ATP (fig.1A). In this experiment the ATP yield per flash was about 1 nmol ATP/mg chl. Assuming a photosynthetic unit size of 500 chl molecules this means 0.5 molecule ATP is formed in every electron transport chain per flash. Considering that there is 1 CF_1 for every 1000 chlorophyll molecules [13] this means that with each flash 1 ATP molecule is formed for every CF_1 which means optimal ATP formation. Since the flash-induced luminescence response was found to be completely abolished by the addition of 4 μM CCCP (fig.1A) the luminescence change undoubtedly reflects ATP formation.

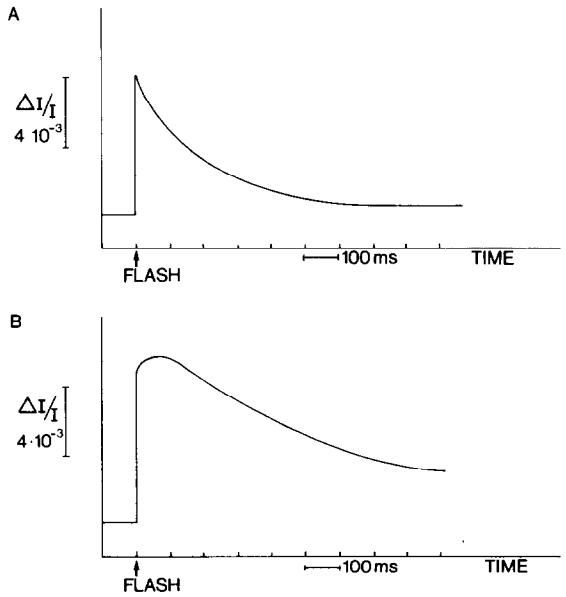

Fig.2. Flash-induced absorbance change at 518 nm in chloroplasts determined after illumination with 4 successive flashes (repetition rate 3 Hz) in chloroplasts isolated from a summer type (A) and a winter type (B) spinach. The (smoothed) curves were obtained after averaging the response of 8 saturating flashes (repetition rate 0.05 Hz).

From fig.1B (right-hand side) it can be seen that in winter chloroplasts, where adenylate kinase activity is much lower than in summer chloroplasts, the first 4 activating flashes did not cause a change in the luminescence signal, i.e. a change in ATP concentration. The same was found for 0.05 Hz repetitive illumination with series of 4 activating flashes (not shown). ATP hydrolysis in these chloroplasts could only be induced by a series of at least 60 flashes or by a 30–60 s period of continuous pre-illumination. Once hydrolysis was induced, flash-induced ATP synthesis could be measured. Under these conditions the yield of ATP per flash was equal to the yield reported for summer chloroplasts (fig.1B). From fig.2B it can be seen that the kinetics of the flash-induced P515 bandshift determined in winter chloroplasts after one series of 4 activating flashes is similar to those normally found in dark-adapted chloroplasts, i.e. there is a pronounced contribution of the slow phase. This result is in agreement with the observation that in these chloroplasts the ATPase cannot be activated by short periods of illumination.

These experiments show that the light energy requirement for the activation of the chloroplast ATPase is different in samples that show a difference in adenylate kinase activity. High activity of the enzyme, as found in our summer type chloroplasts, results in conditions in which ATP hydrolysis could be provoked by as little as 4 activating flashes. Chloroplasts with a low activity of the enzyme require substantially longer periods of illumination for the activation of ATPase. Activation of the ATPase resulted in dark ATP hydrolysis and sustained energization of the membrane in both samples. The latter is indicated by a pronounced alteration in the kinetics of the flash-induced P515 electrochromic bandshift [14]. Under pre-energized conditions flash-induced ATP synthesis was found to be similar in both samples and reached an optimal value of about 1 molecule ATP formed/CF_1 per flash. These results strongly suggest that factors determining ΔG_{ATP} in the dark (e.g. adenylate kinase activity) must be taken into account when kinetic studies of flash-induced ATP synthesis are made. This has been insufficiently considered in the past. The data illustrate the usefulness of the flash-induced P515 response as a qualifying monitoring probe for the energy requirement and maintenance of the activity of the membrane ATPase.

The procedure so far followed for the determination of the P515 response in general includes signal averaging, in order to obtain a sufficient signal-to-noise ratio. It is likely that in plant material with a high adenylate kinase activity the first flashes of a series used for the averaging procedure result in activation of the chloroplast ATPase. The subsequent hydrolysing activity of the ATPase will result in energization of the thylakoid membrane and as a consequence, the P515 response in subsequent flashes is devoid of the slow phase. Consequently the averaged P515 signal then hardly shows, if at all, a contribution of the slow phase (reaction 2). These results explain the fact that in the summer spinach variety grown under sub-optimal light conditions (e.g. with lower light fluence in winter time) the flash-induced 515 nm electrochromic bandshift determined in intact leaves is found to be devoid of the slow phase. This absence of the slow phase in the P515 signal is a rather common seasonal phenomenon, which has plagued our experiments for a long time. It remains to be elucidated whether the adenylate kinase activity in the plant leaves is dependent on the light conditions under which the plants are grown.

With improved measuring techniques one is now able to reach a good definition of the P515 response with a single flash activation. This enables one to discriminate whether the absence of the slow phase in the P515 signal is due to altered membrane constitution [19], or to sustained membrane energization.

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